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Selective peroxisome degradation in *Yarrowia lipolytica* after a shift of cells from acetate/oleate/ethylamine into glucose/ammonium sulfate-containing media

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Abstract We have shown that peroxisomes of the yeast *Yarrowia lipolytica* are subject to specific degradation after exposure of acetate/oleate-grown cells to glucose excess conditions. Electron microscopic analysis has revealed that the peroxisomes were degraded by uptake in the vacuole. Our results suggest that peroxisomes are taken up by macroautophagic processes, because sequestration of individual peroxisomes, which occurs typically at the beginning of microautophagy, was never observed. The observation that a peroxisomal amine oxidase activity is specifically induced by ethylamine was used for the development of a plate assay screening procedure to isolate peroxisome degradation-defective mutants.

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Key words: Yeast; Microbody; Autophagy; Peroxisome degradation; Mutant isolation; *Yarrowia lipolytica*

1. Introduction

Peroxisomes are ubiquitous, essential cell organelles with a remarkable versatility in function which varies with the organism in which they occur [1–3]. Characteristic for peroxisomes is their inducibility in response to external stimuli. In spite of their simple architecture, the biogenesis of these organelles is a complex process involving at least 20 proteins, collectively called peroxins and which are encoded by *PEX* genes [4,5].

Yeasts have been shown to be excellent model organisms for studies on peroxisome biogenesis and much of the current knowledge on peroxisome formation is based on the analysis of yeast *pex* mutants. In yeasts peroxisomes are strongly induced during growth of cells on a number of unusual carbon sources, using for growth e.g. alkanes, methanol, fatty acids, primary amines, D-amino acids and purines [1,6–10]. Under these conditions the organelles are essential for growth as they contain the key enzymes involved in the primary metabolism of these compounds [1]. Remarkably, the opposite may also occur. After a shift of cells from peroxisome-inducing conditions into media in which the organelles are redundant for growth, the peroxisomal population in the cells is rapidly degraded by an autophagic process [9,11–18]. This phenomenon is particularly prominent in methylotrophic yeasts, and recently the first mutants affected in selective peroxisome turn-

over in *Hansenula polymorpha* (pdd mutants) and *Pichia pastoris* (pag mutants) have been isolated [17,18].

The aim of the present study was to make an inventory of the conditions that would lead to selective peroxisome degradation in the yeast *Yarrowia lipolytica*, which grows very well on alkanes and fatty acids but not on methanol. Our data indicated that peroxisome degradation occurred after exposure of acetate/oleate-grown cells to glucose excess conditions. We also observed that *Y. lipolytica* was able to utilize ethylamine as sole nitrogen source; during growth of cells under these conditions a peroxisomal amine oxidase activity was induced. The presence of this enzyme was subsequently applied to develop a screening procedure for the isolation of peroxisome degradation-deficient (pdd) mutants of *Y. lipolytica*, by adapting the plate activity assay developed for monitoring alcohol oxidase activity in colonies of methanol-grown *H. polymorpha*.

2. Materials and methods

2.1. Microorganism and cultivation

The yeast strain used in this study was *Y. lipolytica* PO1d (*MATA leu2-270 ura3-302 xpr2-322* [19]). The organism was grown at 28°C in complete (YEPD) or minimal (YND, YNO) media. YEPD medium contained 1% yeast extract, 2% peptone, 2% glucose; YND contained 0.67% yeast nitrogen base without amino acids, 0.5% glucose and 0.2% ammonium sulfate or 0.2% ethylamine-HCl as nitrogen source. YNO medium contained 0.67% YNB, 0.4% sodium acetate, 0.5% oleic acid, 0.1% Tween 20 and either 0.2% ammonium sulfate or 0.2% ethylamine-HCl as the nitrogen source. If required, leucine was added at 50 mg/l and uracil was added at 20 mg/l.

2.2. Biochemical methods

Crude extracts were prepared as described previously [20]. Amine oxidase (EC 1.4.3.4) and cytochrome *c* oxidase (EC 1.9.3.1) were assayed according to [21]. Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as a standard. SDS-PAGE was carried out as described [22], Western blotting was performed according to [23]. The blots were decorated using polyclonal antibodies against *Y. lipolytica* thiolase and isocitrate lyase protein as well as antibodies against the GroEL protein of *Escherichia coli*, which showed cross-reactivity against a mitochondrial hsp60 homolog of *Y. lipolytica*.

2.3. Mutant isolation procedures

The mutagenesis of cells and subsequent nystatin enrichment were carried out essentially as described [24]. Typically, 50 µl of the frozen mutant stock was diluted to 5 ml with water and 100 µl of this cell suspension was plated onto YEPD. The colonies (approximately 200/plate) were replica plated onto YND and subsequently onto induction medium plates, containing 0.4% acetate, 0.5% oleic acid and 0.2% ethylamine. After incubation for 36 h, the plates were carefully overlaid with 5–10 ml liquid glucose YND medium; after 6–8 h of incu-

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bation the YND was removed and the plates were overlaid with the amine oxidase activity assay mixture, as described by [17], using 0.05% digitonin to permeabilize cells and 2 mM ethylamine instead of methanol as the amine oxidase substrate. Reddish colored colonies due to the presence of amine oxidase activity, representing putative pdd mutants, were screened after 5–6 h of incubation; wild type colonies, grown as controls on the same plates, were invariably unstained after this procedure.

2.4. Electron microscopy

Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously [20]. Immunocytochemistry was performed on ultrathin sections of uncryl-embedded cells, using specific antibodies against *Y. lipolytica* thiolase and isocitrate lyase and gold-conjugated goat anti-rabbit (GAR-gold) antibodies.

3. Results and discussion

3.1. Induction of amine oxidase activity

Cells of *Y. lipolytica* PO1d grew well on YEPD and YND media. Under these conditions the cells generally contained few very small peroxisomes (Fig. 1A).

Several organic nitrogen sources that are known to induce peroxisomal oxidases in various yeasts were tested for induction of peroxisomal oxidase activity. Unfortunately, the *Y. lipolytica* strain used could not grow on D-amino acids and uric acid, and induction of the corresponding redox enzymes,

namely D-amino acid oxidase and uric acid oxidase, was not observed (data not shown). However, when ethylamine was used as sole nitrogen source cells grew normally. Therefore cells from a YND culture, grown in batch culture to a density of $OD_{660} = 3.5$, were diluted 10-fold in fresh media containing ethylamine as the sole nitrogen source. The induction of amine oxidase activity during growth of cells in glucose/ethylamine-containing media is shown in Fig. 2. As is evident from this figure, a normal exponential growth curve was observed. Amine oxidase activity was induced after a lag phase of generally 3–5 h and reached its maximum value at the mid-exponential growth phase and gradually declined thereafter. These induction profiles as well as the specific amine oxidase activities were largely comparable to those observed during growth of other yeasts on primary amines as sole nitrogen source [8]. Compared to glucose/ammonium sulfate-grown cells, the microbodies in cells grown on glucose/ethylamine were enhanced in size but not in number (data not shown).

3.2. Identification of growth parameters to induce selective peroxisome degradation in *Y. lipolytica* PO1d

Previous experiments have made clear that shifting cells from an organic nitrogen source that require peroxisomal oxidase activities for growth (e.g. ethylamine) to conditions in which the synthesis of the enzyme is fully repressed does not

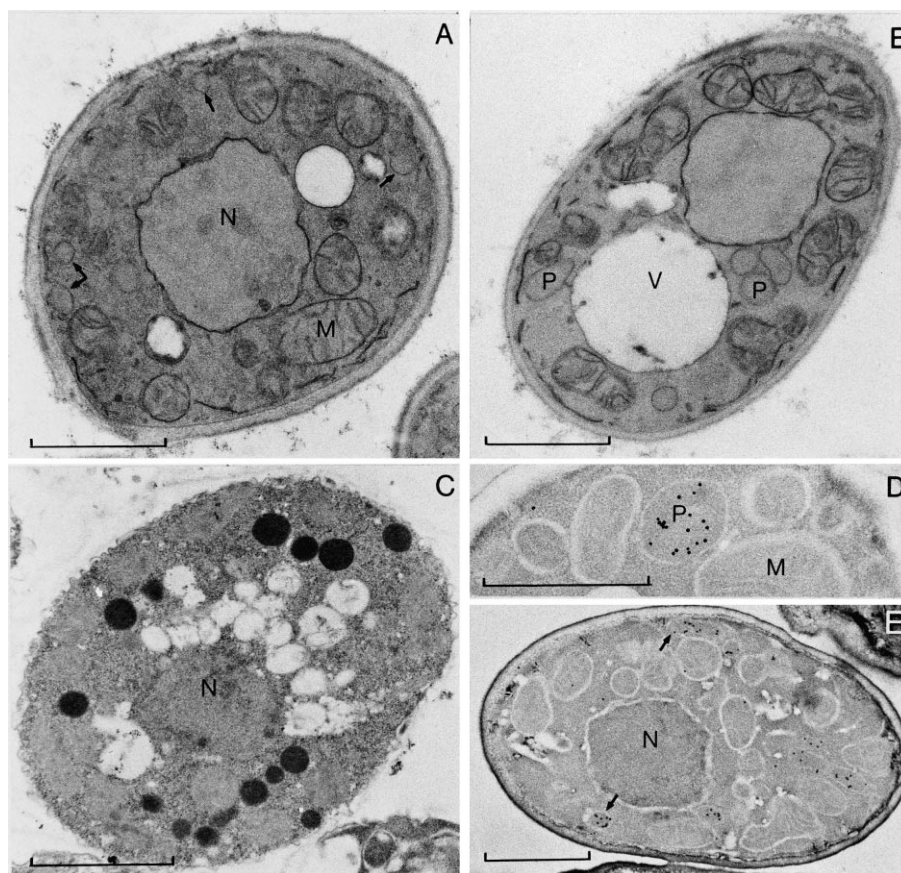


Fig. 1. Electron micrographs of $KMnO_4$ - (A,B), glutaraldehyde/ OsO_4 - (C), or glutaraldehyde/uranylacetate-fixed cells of *Y. lipolytica* showing overall cell morphology and detection of peroxisomal proteins. A: Cells grown on glucose containing only few small peroxisomes (arrows). B: Cells grown on acetate containing larger peroxisomes. C: Protoplasts of cells grown on acetate/oleic acid/ethylamine were incubated with $CeCl_3$ and ethylamine as the amine oxidase substrate. Peroxisomes are stained black by activity of ethylamine oxidase. D,E: Characteristic labeling pattern after immunocytochemical experiments on oleic acid/ethylamine-grown cells, using anti-thiolase (D) and anti-ICL antibodies (E). The labeling is confined to peroxisomal profiles, from which some are indicated by arrows in the survey of E. M = mitochondrion, N = nucleus, P = peroxisome, V = vacuole. The marker represents 0.5 μm .

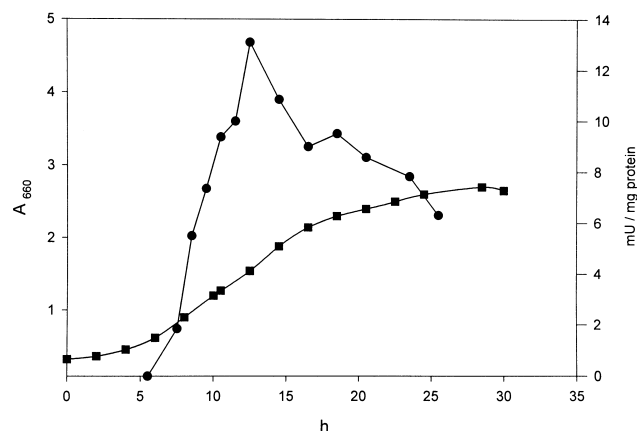


Fig. 2. Growth of *Y. lipolytica* PO1d (■) and induction of amine oxidase activity (●) after transfer of glucose/ammonium sulfate-grown cells into glucose/ethylamine-containing medium.

lead to peroxisome degradation. Invariably, the observed enzyme inactivation could be accounted for by dilution of enzyme protein over newly formed cells.

In contrast, a strong selective degradation is induced after a shift of methylotrophic yeasts from methanol – in which peroxisomes are massively induced – into glucose-containing media [1,9,11,14]. However, since *Y. lipolytica* does not grow on methanol, we decided to search for other growth conditions that lead to a strong microbody proliferation. Highest peroxisome induction rates were observed during growth of cells on acetate/oleic acid-containing media (Fig. 1B), compared to growth on acetate or oleic acid alone (not shown). In ultrathin sections of acetate/oleic acid-grown cells frequently over 12 peroxisomal profiles were observed. Identical numbers of organelles were observed in cells grown on mixtures of acetate/oleic acid in the presence of ethylamine as sole nitrogen source (Fig. 1C). In such cells the organelles frequently occurred in small clusters, consisting of 2–4 organelles, which were randomly distributed over the cytoplasm. Immunocytochemically, the organelles were characterized by the presence of thiolase (Fig. 1D), isocitrate lyase (not shown) and amine oxidase activity (Fig. 1C).

Exposure of acetate/oleic acid/ethylamine-grown cells to glucose excess led to a rapid inactivation of amine oxidase

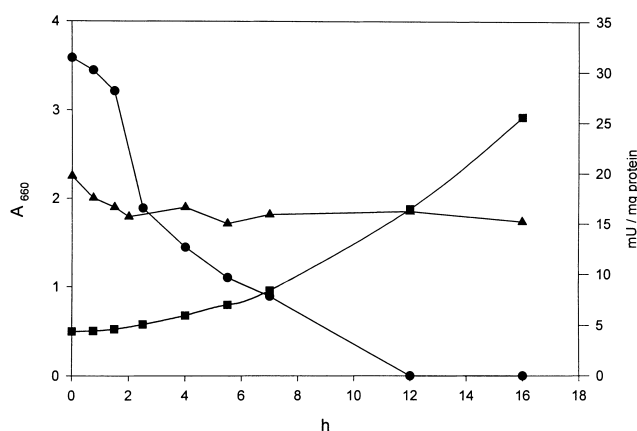


Fig. 3. Growth curve (■) and activities of ethylamine oxidase (●) and cytochrome *c* oxidase (▲) in cells shifted from acetate/oleic acid/ethylamine- to glucose/ammonium sulfate-containing medium.

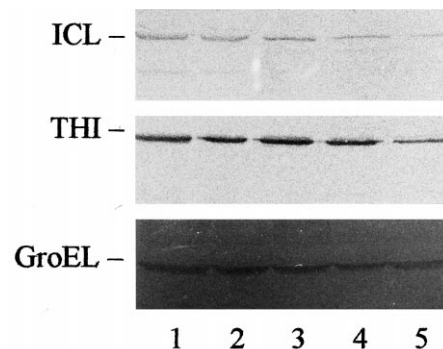


Fig. 4. Degradation of isocitrate lyase and thiolase protein after a shift of acetate/oleic acid/ethylamine-grown cells into fresh glucose/ammonium sulfate-containing media. Western blots were prepared of crude extracts of cells, grown for 1, 2, 3, 4 or 5 h in the new environment. The blots were decorated using anti-isocitrate lyase (ICL), anti-thiolase (THI) antisera and anti-GroEL antisera. 10 μ g protein was loaded on each lane.

activity (Fig. 3). Cytochrome *c* oxidase activities, measured as a marker enzyme for mitochondria, remained approximately constant in the same time interval.

Since specific antibodies against *Y. lipolytica* amine oxidase were not available, we could not discriminate whether the amine oxidase was due to enzyme modification or to the degradation of enzyme protein. The latter aspect was studied using specific antibodies against *Y. lipolytica* thiolase. Western blots, prepared from crude extracts of samples taken after various time points following the addition of glucose to the culture, clearly demonstrated that the reduction in thiolase protein was due to enzyme protein degradation (Fig. 4). Similar degradation patterns were observed for isocitrate lyase (Fig. 4). As a control mitochondrial cytochrome *c* oxidase activities were measured, which remained constant in this time interval. Additionally, antibodies against bacterial GroEL, which exhibited cross-reactivity against a hsp60 homolog of *Y. lipolytica*, were used to show that mitochondrial proteins were not degraded in the same time interval (Fig. 4).

Since thiolase and amine oxidase were in the same organelle, judged from electron microscopy, this led us to conclude that the decrease in amine oxidase activity is due to the disappearance of amine oxidase protein due to the degradation of peroxisomes. This suggestion was furthermore confirmed after electron microscopic analysis of the various samples which revealed that the organelles were degraded by uptake in the vacuole (Fig. 5A,B). The mechanisms of the vacuolar uptake are not yet clear. However, since sequestration of individual peroxisomes, a phenomenon illustrative for the first step of microphagic degradation in methylotrophic yeasts [9,12,18], was never observed, we assume that in *Y. lipolytica* peroxisomes are degraded by macroautophagic processes, as described for *Pichia pastoris* after transfer of cells from methanol to ethanol media [18].

3.3. Isolation of mutants affected in peroxisome degradation

The established procedure to induce peroxisome degradation in *Y. lipolytica* was subsequently used to adapt the plate activity assay, developed for the isolation of pdd mutants in *H. polymorpha*. Mutagenized cells (for details see Section 2) were grown on solid agar media supplemented with 0.4% acetate and 0.1% oleic acid as carbon sources in the presence of

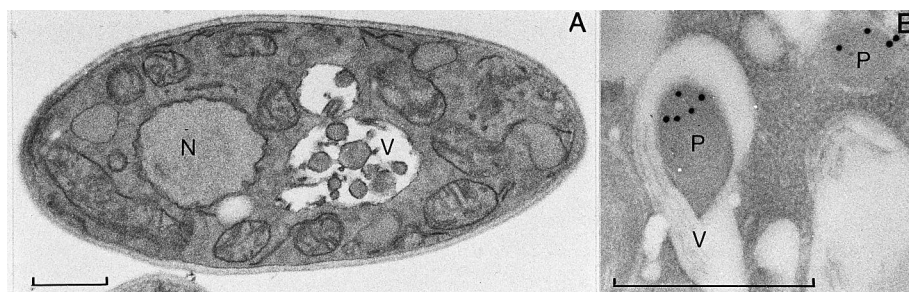


Fig. 5. Electron micrographs taken from KMnO_4 -fixed cells shifted from acetate/oleic acid/ethylamine- into fresh glucose/ammonium sulfate-containing media. The accumulation of cell constituents in the vacuole of cells is clearly seen in A. These particles were identified as peroxisomes after immunocytochemical staining procedures, using anti-isocitrate antibodies (B).

0.2% ethylamine as the nitrogen source. After incubation for 36 h the plates were overlaid with 6 ml of the liquid amine oxidase activity assay mixture, containing 2% agar, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 0.05% digitonin and 2 mM ethylamine as the amine oxidase substrate. After solidification the plates were stored at 28°C and examined at regular time intervals. The results indicated that after 6 h a distinct coloring was evident of all individual colonies, which was absent in control plates lacking the amine substrate and on plates which contained ammonium sulfate instead of ethylamine as nitrogen source. This indicates that the method is suitable to monitor amine oxidase activity in colonies of *Y. lipolytica*, grown in the presence of primary amines as sole nitrogen source.

Subsequent experiments to isolate pdd mutants by this method revealed that replica plating to fresh glucose plates to induce peroxisome degradation in the acetate/oleate/ethylamine-grown cells gave unreproducible results due to the fact that frequently insufficient material could be replicated to give a clear-cut amine oxidase activity response. Therefore, we decided to adapt the method and expose the colonies, developed on acetate/oleate/ethylamine plates, to excess glucose by overlaying the plates with 6 ml of liquid glucose medium. After incubation for 6 h at 28°C this medium was carefully removed and replaced by the amine oxidase activity assay mixture. The plates were screened for colored colonies after 6–7 h of incubation of the plates at 28°C. On average, 1–3 colonies were present per plate (containing 50–60 colonies) that displayed a coloring intensity that was identical to untreated controls. Also, WT colonies that were present on each plate as positive control invariably remained fully unstained after this procedure. By this way, we could identify approximately 50 putative pdd strains. The selected strains were all re-checked in a next round of growth and peroxisome degradation analysis, which led to the isolation of 48 strains that all met the criteria for putative pdd mutants. At present, the mutants are subjected to further genetic (e.g. complementation analysis) and biochemical analyses to confirm their pdd phenotype. Selected mutants will subsequently be used for cloning and characterization of the corresponding genes.

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